Ecotoxicology and Environmental Safety 147 (2018) 954–962

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Biochemical responses and accumulation patterns of *Mytilus galloprovincialis* exposed to thermal stress and Arsenic contamination

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ARTICLE INFO

Keywords:
Climate change
Metalloids
Bioaccumulation
Oxidative stress
Metabolism
Mussels

ABSTRACT

Organisms in marine systems are exposed to multiple stressors that create a range of associated environmental and ecotoxicological risks. Examples of stressors include alterations related to climate change, such as temperature increase, and the exposure to pollutants arising from human activities. The present study evaluated the impacts of Arsenic exposure (1 mg/L) and warming (21 °C) in *Mytilus galloprovincialis*, acting alone and in combination. Our results demonstrated that both Arsenic exposure and warming induced oxidative stress and reduced mussels metabolism, with changes becoming more prominent with the exposure time and when mussels were exposed to both stressors in combination. Furthermore, results obtained showed higher As accumulation in organisms exposed to warming treatments. The present study showed that under warming scenarios, the negative impacts induced by As may be enhanced in ecologically and economically relevant bivalves, with potential impacts on population stocks due to increased sensitivity to pollutants, which may eventually result in biodiversity loss and socio-economic impacts.

1. Introduction

Increasing CO_2 concentrations in the atmosphere, which result from fossil fuel combustion and industrial processes, have led to rising air temperature and consequently to oceans warming (IPCC, 2014). The global average surface temperature has increased, particularly since the 1950s, with an average warming rate over the last 50 years of 0.13 °C ± 0.03 °C per decade, nearly twice that of the past century (IPCC, 2007). According to recent IPCC predictions, surface water temperatures are expected to increase in the next decades between 1.4 °C and 3.1 °C or between 2.6 °C and 4.8 °C (RCP 6.0 and RCP 8.5 IPCC scenarios, respectively; IPCC, 2014). In the aquatic environment, invertebrates are particularly sensitive to thermal stress because of their ectothermic biology (e.g. Pörtner, 2010). Studies already demonstrated that temperatures that exceed the organisms' thermal tolerance range are responsible for physiological and molecular perturbations, including lowered scope for growth and reproduction (Pörtner and Knust, 2007; Boukadida et al., 2016), decreased aerobic capacity, metabolic rate and respiratory capacity (Jansen et al., 2009; Pörtner, 2002a, 2002b, 2010, 2005; Velez et al., 2017). Warming is also known to enhance reactive oxygen species (ROS) production in cells (Kefaloyianni et al., 2005; Verlecar et al., 2007) thereby increasing the risk of oxidative alteration. The induction of oxidative stress was already observed in bivalves exposed to warming conditions, including oysters (*Crassostrea gigas, C. brasiliana*), clams (*Ruditapes philippinarum, R. decussatus*) and mussels (*Mytilus galloprovincialis*) (Attig et al., 2014; Banni et al., 2014a; Hu et al., 2015; Nardi et al., 2017; Velez et al., 2017).

Anthropogenic activities may not only result in climate change but have also been the cause of increasing concentrations of pollutants in the aquatic environments, threatening inhabiting organisms. Coastal areas, namely estuaries and lagoons, are often the final destination of pollutants, including metals and metalloids, which tend to be accumulated not only in the sediments but also by the organisms inhabiting these areas, namely bivalves due to their filter-feeding and sedentary behaviour (Zhang et al., 2015, 2012; Ventura-Lima et al., 2009, 2011), with associated toxic effects (Ahmad et al., 2011; Fattorini et al., 2004, 2006; Freitas et al., 2012; Velez et al., 2015a, 2015b). Arsenic (As) is a metalloid that is widely distributed in the aquatic environment mainly in its inorganic and more toxic forms (arsenite and arsenate) (Fattorini and Regoli, 2004). The range of As concentrations found in natural waters varies from 0.5 µg/L to more than 5 mg/L (Smedley and Kinniburgh, 2002; Velez et al., 2014; 2015a) while in sediment

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http://dx.doi.org/10.1016/j.ecoenv.2017.09.051
Received 10 July 2017; Received in revised form 15 September 2017; Accepted 17 September 2017
Available online 05 October 2017
0147-6513/

concentrations may reach 3000 mg/kg (Mandal and Suzuki, 2002). Reported values for As concentrations accumulated by several estuarine bivalve species range from 4 to 40 mg/kg DW in *Mytilus galloprovincialis* mussels (Argese et al., 2005; Belivermiş et al., 2016; Orescanin et al., 2006), 7 to 14 mg/kg FW in *Ruditapes decussatus* and *R. philippinarum* clams (Velez et al., 2015b), and 1.1 to 2.4 mg/kg FW in *Cerastoderma edule* cockles (Freitas et al., 2012, 2014). As is listed as the most high-priority hazardous substance in the world (ATSDR, 2015) and the toxicity induced by this metalloid has been related to changes in cellular oxidative stress balance, often leading to a prooxidant state (Company et al., 2004; Samuel et al., 2005; Ventura-Lima et al., 2011), including in bivalves, namely in mussels (*M. galloprovincialis*), clams (*R. philippinarum, R. decussatus*) and oysters (*C. gigas, C. angulata*) (Freitas et al., 2016a, 2016b; Mejdoub et al., 2017; Moreira et al., 2016a, 2016b; Richir and Gobert, 2014; Velez et al., 2016c).

Among marine bivalve species, the Mediterranean mussel *M. galloprovincialis* is a biological resource with high economic value, commonly used in ecotoxicological studies to assess environmental pollution levels and impacts. Due to their wide distribution and abundance, sedentary lifestyle, tolerance to a wide range of environmental conditions, this species has been widely used as a sentinel and bioindicator (among others, Wang et al., 1996; Banni et al., 2014a, 2014b; Viarengo et al., 2007). Sedentary filter–feeder mussels, such as *M. galloprovincialis*, have a large capacity to accumulate pollutants in their tissues leading to a toxic response (Livingstone et al., 2000; Catsiki and Florou, 2006; Gielazyn et al., 2003). Biochemical and physiological responses of *M. galloprovincialis* adults and juveniles to environmental pollutants are well documented (e.g. LeBlanc et al., 2005; Attig et al., 2014; Banni et al., 2014a, 2014b; Benali et al., 2016; Boukadida et al., 2017). Studies with *M. galloprovincialis* demonstrated that this species is negatively affected by metals, with alterations of mussels' oxidative status (Banni et al., 2014a; Benali et al., 2016; Boukadida et al., 2017; Mejdoub et al., 2017), proteomic and metabolomic responses of larvae and juveniles (Wu et al., 2016; Yu et al., 2016), lysosomal biomarkers and gene transcription (Izagirre et al., 2014). More recently, studies have also shown the capacity of this species to respond to climate change related factors revealing metabolic, physiological (e.g. clearance rate, oxygen consumption rate, excretion rate) and oxidative status alterations due to ocean acidification, salinity alterations and warming (Anestis et al., 2010; Attig et al., 2014; Banni et al., 2014a; Boukadida et al., 2016; Fearman and Moltschaniwskyj, 2010; Izagirre et al., 2014; Jansen et al., 2009; Zittier et al., 2015).

Although it has been reported that warming and pollutants induce negative impacts to aquatic organisms, including bivalves, scarce information is available regarding the impacts of thermal stress on the sensitivity of organisms towards pollutants as well as regarding changes in pollutants toxicity (Attig et al., 2014; Banni et al., 2014a, 2014b; Izagirre et al., 2014). Particularly, while an increasing body of knowledge has demonstrated the physiological and biochemical impacts induced by warming and As in bivalves (see references above), no information is known on the alterations caused in *M. galloprovincialis* by both stressors acting in combination. For this reason, in the present study mussels were exposed for 28 days to As and warming, acting alone and in combination, and As accumulation patterns as well as biomarkers related to oxidative stress and metabolic capacity were evaluated.

2. Materials and methods

2.1. Experimental conditions

Mytilus galloprovincialis specimens were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in September 2016. In order to minimize the effect of body weight, organisms with similar weight (21.3 ± 6.6 g) were selected.

Individuals were maintained in the laboratory for 15 days before testing, in separate aquaria, in order to release metals and microorganisms (Freitas et al., 2012; Maffei et al., 2009). During this period, organisms were maintained at 17.0 ± 1.0 °C; pH 7.80 ± 0.10, 12 h light: 12 h dark photoperiod and continuous aeration, in artificial seawater (salinity 35 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center). Seawater was renewed every two days.

After this period, organisms were distributed into different glass containers (3 L seawater, salinity 35), with 3 individuals per container and 8 containers per treatment. The treatments tested were: control temperature (17 °C) without As: 17 °C, corresponding to control treatment; control temperature (17 °C) with As 1.0 mg/L: As 17 °C; temperature 21 °C without As: 21 °C; temperature 21 °C with As 1.0 mg/L: As 21 °C. To maintain the temperature levels (17 and 21 °C) containers were placed in different climatic chambers.

Taking into consideration preliminary experiments conducted by the team (this study, data not shown; Velez et al., 2016c; Velez et al., 2016a), As was dosed at a sublethal concentration of 1.0 mg/L, using Sodium Arsenate (Sigma-Aldrich), which enabled mussels to accumulate As concentrations in the range reported in bivalves at the Ria de Aveiro, but also values recorded in bivalves from other estuarine systems (Argese et al., 2005; Belivermiş et al., 2016; Freitas et al., 2012, 2014; Orescanin et al., 2006; Velez et al., 2014, 2015a).

Taking into account the average temperature of the sampling area during September (16–19 °C, IPMA, 2016) 17 °C was selected as control temperature. Considering annual range of average temperatures (13.4–22.9 °C) for *M. galloprovincialis* habitats in the Ria de Aveiro (Coelho et al., 2014; Santos et al., 2009; Velez et al., 2015a) and the predicted temperature increase from 1.0 to 4.0 °C (IPCC, 2007), 21 °C was selected to represent warming conditions.

The experiment was conducted over 28 days, and organisms were collected 14 (12 per treatment) and 28 (12 per treatment) days after the start of exposure. During the exposure period containers were continuously aerated, temperature and salinity were daily checked with a thermometer and a refractometer, respectively. When necessary, temperature and salinity were adjusted adding water to the containers, according to the above conditions. Mortality was daily checked and organisms were considered dead when their shells gaped and failed to shut again after external stimulus.

During the entire exposure period (28 days) animals were fed with Algamac protein plus (150.000 cells/animal) twice a week and exposure medium (seawater at salinity 35) was renewed weekly, after which As concentration was reestablished.

At the end of the exposure, organisms were frozen individually and manually homogenized with a mortar and a pestle in liquid nitrogen. Each homogenized organism was divided into 0.5 g aliquots, used for biomarker analyses and As quantification.

2.2. Arsenic quantification

Arsenic concentrations in *M. galloprovincialis* were determined by inductively coupled plasma mass spectroscopy (ICP-MS), after microwave assisted digestion. Freeze-dried samples (100–200 mg) were digested in a CEM MARS 5 microwave, first with 2 mL HNO_3 (70%) for 15 min at 170 °C, followed by a second microwave cycle with 0.5 mL H_2O_2 (30%) for 15 min at 170 °C. After addition of H_2O_2, the mixture was left to sit for 15 min to allow any gas to vent, before the reaction vessels were tightened and placed in the microwave. The obtained digests were transferred into 25 mL polyethylene vessels and the volume made up with ultrapure water.

As quantification in samples by ICP-MS was performed on a Thermo ICP-MS XSeries equipped with a Burgener nebuliser. The limit of quantification of the method was 1 μg/L, with an acceptable coefficient of variation among replicates of 5%.

The quality control was assured by running procedural blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material TORT-2 (*Lobster Hepatopancreas*; 21.6 ± 1.8 mg As kg^{-1}) in

parallel with samples. Blanks were always below the quantification limit and mean percentage of recovery for As was 110 ± 4% (n = 4).

2.3. Biomarkers

Biomarkers were determined in organisms whole soft tissue. For each biochemical determination, 0.5 g fresh weight (FW) soft tissue per organism was used (3 individuals per replicate and 12 per treatment, 48 per sampling period). For each treatment, energy-related (total protein content, PROT; glycogen content, GLY; and electron transport system activity, ETS), and oxidative stress (superoxide dismutase activity, SOD; catalase activity, CAT; glutathione S-transferases activity, GSTs; lipid peroxidation levels, LPO; and oxidized (GSSG) glutathione) markers were determined. All biochemical parameters were performed in duplicate. All measurements were made using a microplate reader (Biotek). The extraction for each biomarker was performed with specific buffers. Samples were homogenized for 15 s at 4 °C and centrifuged for 10 min at 10,000*g* (or 3000*g* for ETS) and 4 °C. Supernatants were stored at − 80 °C or immediately used.

For LPO assay supernatants were extracted in 20% (v/v) trichloroacetic acid (TCA) (1:2, w/v). For GSSG assay samples were extracted in 0.1 M dipotassium phosphate, with 5 mM EDTA, 0.1% Triton X-100, pH 7.5. For SOD, CAT, GSTs, PROT and GLY assays supernatants were extracted in phosphate buffer (50 mM sodium dihydrogen phosphate monohydrate; 50 mM disodium hydrogen phosphate dehydrate; 1 mM ethylenediamine tetra-acetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) (PVP); 1 mM dithiothreitol (DTT) (pH 7.0). For the ETS activity assay supernatants were extracted in 0.1 M Tris–HCl buffer (15% (w/v) polyvinylpyrrolidone (PVP); 153 mM magnesium sulfate (MgSO4); 0.2% (v/v) Triton X-100) (pH 8.5).

2.3.1. Oxidative stress markers

LPO determination was done following the method described by Ohkawa et al. (1979). LPO levels were measured trough the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 535 nm ($\varepsilon = 156\ mM^{-1}\ cm^{-1}$). LPO levels were expressed in nmol of MDA formed per g of FW.

The quantification of GSSG was performed following the method described in Rahman et al. (2007), using GSSG as standard (0–90 µmol/L). Absorbance was measured at 412 nm and results expressed in µmol per g of FW.

SOD was determined by the method described in Beauchamp and Fridovich (1971) and adaptations performed by Carregosa et al. (2014). The standard curve was determined using SOD standards (0.0001–60 U/mL). Absorbance was measured at 560 nm. The enzymatic activity was expressed in U/g of FW, where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

CAT was quantified according to Johansson and Borg (1988) method and the modifications performed by Carregosa et al. (2014). The standard curve was determined using formaldehyde standards (0–150 µM). Absorbance was measured at 540 nm. The enzymatic activity was expressed in U/g of FW, where U represents the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min at 25 °C.

GSTs activity was quantified following Habig et al. (1974) protocol with some adaptations performed by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). The enzymatic activity was expressed in U/g of FW where U is defined as the amount of enzyme that conjugates 1 µmol of CDNB (1-chloro-2,4-dinitrobenzene) with reduced glutathione (GSH) per min at 25 °C.

2.3.2. Energy-related markers

ETS activity was measured based on King and Packard (1975) and the modifications performed by De Coen and Janssen (1997). The absorbance was measured at 490 nm during 10 min with 25 s intervals. The amount of formazan formed was calculated using $\varepsilon = 15{,}900\ M^{-1}\ cm^{-1}$ and the results expressed in nmol/min/g FW.

For GLY quantification the sulphuric acid method was used, as described by Dubois et al. (1956). Glucose standards were used (0–10 mg/mL). Absorbance was measured at 492 nm. The results were expressed in mg/g FW.

The PROT content was determined according to the spectrophotometric method of Biuret (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used as standard (0–40 mg/mL). Absorbance was measured at 540 nm. The results were expressed in mg/g FW.

2.4. Statistical analyses

Biochemical parameters obtained from each treatment were submitted to statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008).

The null hypotheses tested were: i) for each biochemical marker and sampling period (14 and 28 days), no significant differences exist among treatments (17 °C, As 17 °C, 21 °C, As 21 °C); ii) for each biochemical marker and for each treatment (17 °C, As 17 °C, 21 °C, As 21 °C), no significant differences exist between sampling periods (14 and 28 days).

In figures, lowercase letters represent significant ($p \leq 0.05$) differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 14 days sampling period, while uppercase letters represent differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 28 days sampling period. In figures, for each treatment (17 °C, As 17 °C, 21 °C, As 21 °C) significant differences between sampling periods (14 and 28 days) are presented with asterisks.

3. Results

3.1. Mortality

After 14 days of exposure, none of the tested treatments induced mortality. After 28 days of exposure, no mortality was recorded at control and 21 °C, while 25 (± 32) and 17 (± 19)% of mortality was recorded in mussels exposed to As 17 °C and As 21 °C, respectively.

3.2. Arsenic levels in mussels

Arsenic concentrations in mussels exposed to control (17 °C) and 21 °C treatments were lower than 1.2 µg/g FW, both at days 14 and 28 of experiment and regardless the exposure period, no significant differences were found between organisms exposed to these two treatments (Table 1). Arsenic concentrations in mussels exposed to this metalloid were significantly higher in the As 21 °C treatment than in the As 14 °C treatment, both after 14 and 28 days of exposure (Table 1).

Table 1
Arsenic concentration in mussels (µg/g), at 14 and 28 days after the beginning of the experiment. Concentrations were measured in organisms from different treatments: 17 °C: As 0 mg/L and temperature 17 °C; As 17 °C: As 1.0 mg/L and temperature 17 °C; 21 °C: As 0 mg/L and temperature 21 °C; As 21 °C: As 1.0 mg/L and temperature 21 °C. Different letters represent differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C): lowercase letters represent differences for 14 days sampling period, while uppercase letters represent differences for 28 days sampling period.

	Sampling periods	
	14 days	**28 days**
17 °C	1.11 ± 0.21^{a}	$1.13 \pm 0{,}2.^{A}$
As 17 °C	1.99 ± 0.10^{b}	1.85 ± 0.17^{B}
21 °C	1.02 ± 0.11^{a}	1.15 ± 0.13^{A}
As 21 °C	2.32 ± 0.13^{c}	2.04 ± 0.06^{C}

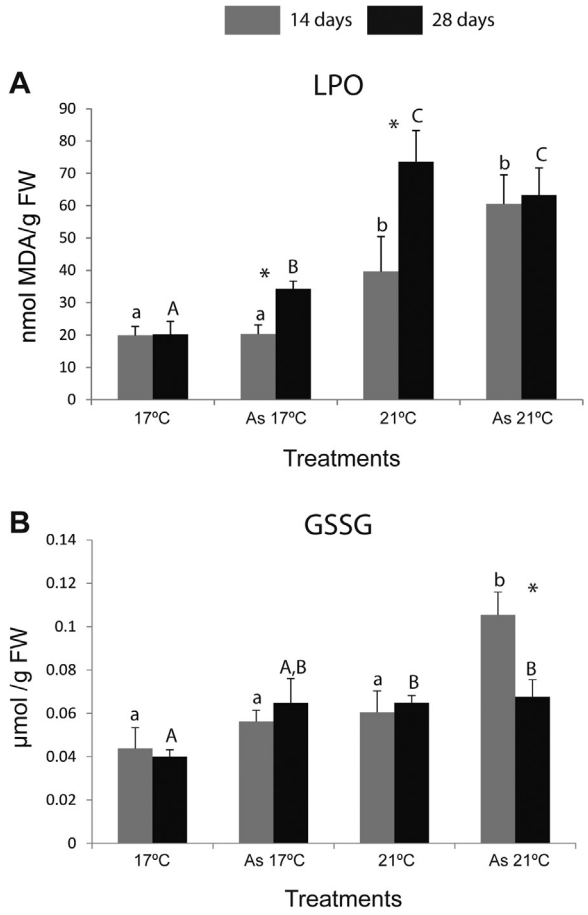

Fig. 1. A: Lipid peroxidation (LPO) levels; **B:** Oxidized glutathione GSSG in *Mytilus galloprovincialis* exposed to different conditions (17 °C, As 17 °C, 21 °C, As 21 °C) after 14 and 28 days of exposure. Results are means + standard deviation. Different lowercase letters represent significant differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 14 days sampling period, while different uppercase letters represent significant differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 28 days sampling period. At each treatment (17 °C, As 17 °C, 21 °C, As 21 °C), significant differences between sampling periods (14 and 28 days) are presented with asterisks.

Comparing sampling periods (14 and 28 days), no significant differences in As concentration was observed for any of the treatments (17 °C, As 17 °C, 21 °C, As 21 °C) (Table 1).

3.3. Biochemical markers

3.3.1. Oxidative stress related markers

After 14 days of exposure, LPO levels significantly increased in organisms exposed to 21 °C and As 21 °C, while in organisms exposed for 28 days LPO also significantly increased in mussels exposed to As 17 °C (Fig. 1A). Significant differences between sampling periods were only observed in organisms exposed to As 17 °C and 21 °C, with higher values in organisms exposed for 28 days (Fig. 1A).

After 14 days of exposure, significantly higher GSSG values were observed between organisms exposed to As 21 °C and the remaining treatments (Fig. 1B). A similar pattern was identified after 28 days of exposure, but in this case significantly higher GSSG values were observed between control and warming treatments (21 °C and As 21 °C) (Fig. 1B). Significant differences between sampling periods were only recorded in organisms exposed to As 21 °C, with the highest values in mussels exposed for 14 days (Fig. 1B).

Concerning SOD activity, mussels showed a similar response after 14 and 28 days of exposure, with no changes among treatments, except in mussels exposed to As 21 °C after 28 days of exposure that presented

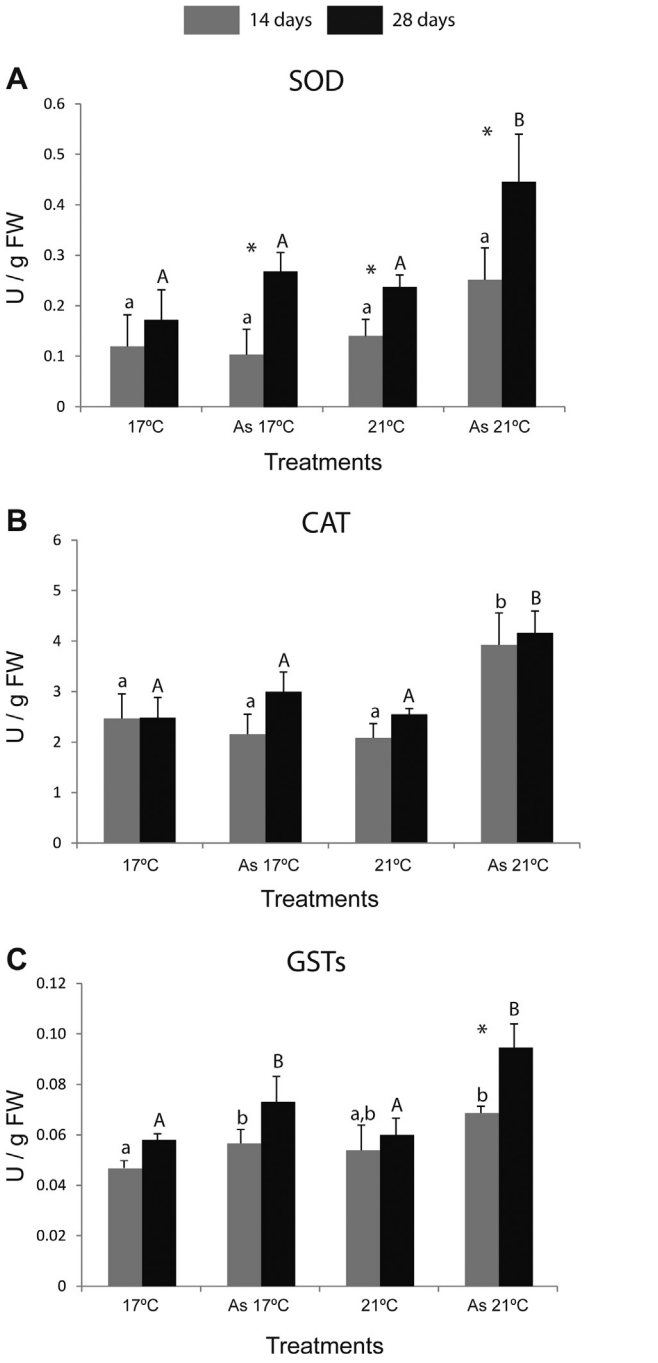

Fig. 2. A: Superoxide dismutase (SOD) activity; **B:** Catalase (CAT) activity; **C:** Glutathione S-transferases (GSTs) activity in *Mytilus galloprovincialis* exposed to different conditions (17 °C, As 17 °C, 21 °C, As 21 °C) after 14 and 28 days of exposure. Results are means + standard deviation. Different lowercase letters represent significant differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 14 days sampling period, while different uppercase letters represent significant differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 28 days sampling period. At each treatment (17 °C, As 17 °C, 21 °C, As 21 °C), significant differences between sampling periods (14 and 28 days) are presented with asterisks.

significantly higher activity compared to the remaining treatments (Fig. 2A). Comparing both exposure periods, significantly higher SOD activity was observed in organisms exposed for 28 days to As 17 °C, 21 °C, As 21 °C treatments (Fig. 2A).

After 14 and 28 days of exposure, the activity of CAT was unchanged in organisms exposed to As 17 °C and 21 °C compared to control organisms, but increased significantly in organisms exposed to As 21 °C (Fig. 2B). No significant differences were observed in CAT

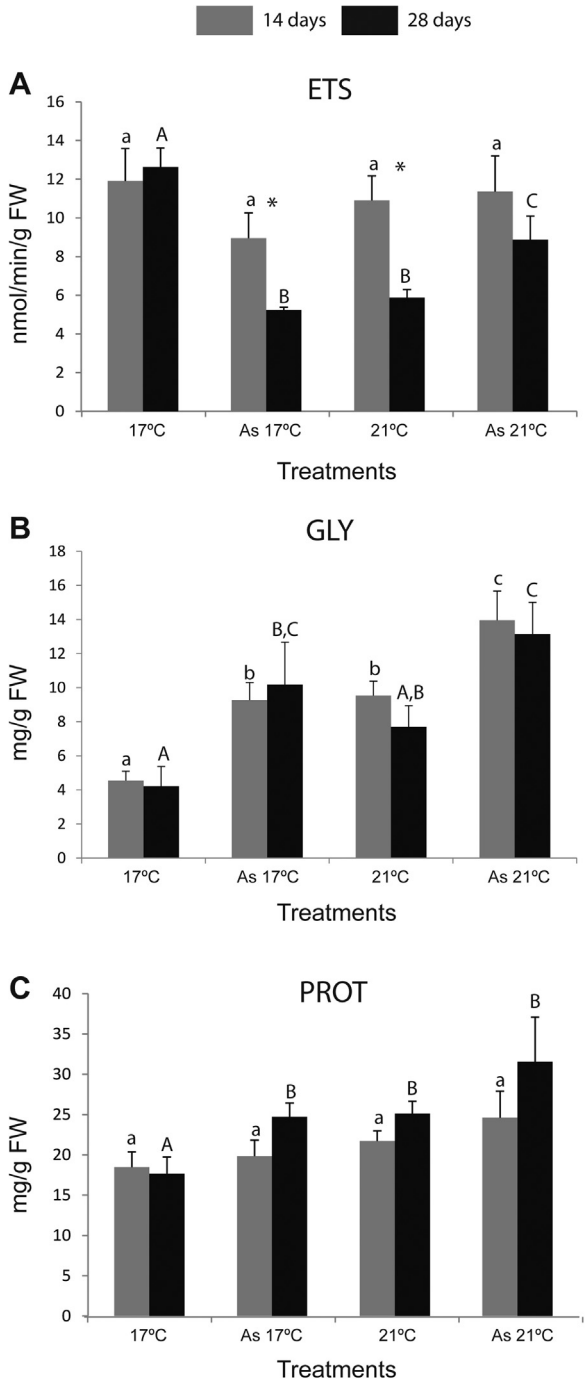

Fig. 3. A: Electron transport system (ETS) activity; **B:** Glycogen (GLY) content; **C:** Protein (PROT) content in *Mytilus galloprovincialis* exposed to different conditions (17 °C, As 17 °C, 21 °C, As 21 °C) after 14 and 28 days of exposure. Results are means + standard deviation. Different lowercase letters represent significant differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 14 days sampling period, while different uppercase letters represent significant differences among treatments (17 °C, As 17 °C, 21 °C, As 21 °C) for 28 days sampling period. At each treatment (17 °C, As 17 °C, 21 °C, As 21 °C), significant differences between sampling periods (14 and 28 days) are presented with asterisks.

activity between exposure periods, regardless of the tested treatment (Fig. 2B).

The activity of GSTs was significantly higher in organisms exposed to As (As 17 °C and As 21 °C) compared to control organisms and 21 °C, both after 14 and 28 days of exposure (Fig. 2C). The GSTs activity in mussels exposed for 14 and 28 days showed no significant differences between 21 °C and control organisms (Fig. 2C). Significant differences between sampling periods were observed in As 21 °C, with the highest values observed after 28 days of exposure (Fig. 2C).

3.3.2. Energy-related parameters

ETS activity was statistically indistinguishable between treatments and controls after 14 days of exposure (Fig. 3A) However, at the end of the experimental period (28 days) mussels exposed to all treatments presented significantly lower ETS activity compared to control individuals (Fig. 3A). When comparing sampling periods significant differences were observed between individuals exposed to As 17 °C and 21 °C, with higher values in mussels exposed for 14 days (Fig. 3A).

Regarding the GLY content, except for organisms exposed for 28 days at 21 °C, after 14 and 28 days of experiment mussels showed significantly higher GLY for the As 17 °C, 21 °C and As 21 °C treatments compared to control (Fig. 3B). When comparing sampling periods, no significant differences were found in GLY content for any of the treatments (Fig. 3B).

After 14 days of exposure, mussels tended to present increased PROT content for the As 17 °C, 21 °C and As 21 °C treatments, but no significant differences were observed among treatments (Fig. 3C). When exposed for a longer period (28 days) a similar pattern was identified but in this case significantly higher PROT content was observed in individuals exposed to As 17 °C, 21 °C and As 21 °C compared to control individuals (Fig. 3C). Comparing both sampling periods (14 and 28 days) no significant differences were obtained regarding PROT content for any of the treatments (Fig. 3C).

4. Discussion

Oxidative stress can take place in marine bivalves under a series of environmental adverse conditions, including thermal stress and contamination by As, which was confirmed in the present study.

The present results demonstrated that contamination by As, warming conditions (21 °C) and the combination of both stressors (As and 21 °C) induced LPO in mussels, especially after long-term exposure (28 days). Our study also indicates that warming induced greater cellular damage in mussels than As contamination as shown by higher LPO levels in mussels exposed to thermal stress compared to mussels exposed to 1 mg/L As. However, our results further demonstrated that when both stressors were acting in combination no additive effects were observed in comparison to thermal impacts. When ROS increase in the cells, scavengers such as reduced glutathione (GSH) can act as antioxidants in the cytoplasm, directly neutralizing several reactive species, being oxidized to GSSG (Regoli and Giuliani, 2014). For this reason, GSSG content has been used as an indication of oxidative stress (e.g. Alves de Almeida et al., 2007). Our study shows that, accompanying the cellular damage pattern, when under stressful conditions mussels increased GSSG content, a response to the excess of ROS production. The highest GSSG content in mussels exposed to the combination of As and increased temperature may have prevented higher LPO levels at this condition. Previous studies also showed that metals and metalloids induced LPO increase in mussels, namely in *M. galloprovincialis* exposed to Ni (Attig et al., 2014), Cd (Nardi et al., 2017), Cu, Cd, Pb and Fe (Vlahogianni and Valavanidis, 2007). Other bivalves also showed similar responses namely clams (*Ruditapes philippinarum, R. decussatus*, *Scrobicularia plana*) exposed to Cu, Zn, Hg, Cd, Pb or As (Ahmad et al., 2011; Freitas et al., 2014; Geret et al., 2003; Geret and Bebianno, 2004; Velez et al., 2016b, 2016c). Regarding the impacts of temperature on LPO, recent studies demonstrated that warming conditions induced cellular damage in *M. galloprovincialis* mussels (Attig et al., 2014) and in *R. philippinarum* clams (Velez et al., 2017). Nevertheless, different patterns were also observed by different authors, which may be related to species specific responses, the element tested, warming conditions, the period of exposure or the organ analyzed. Nardi et al. (2017) showed no significant differences in LPO levels in the digestive glands and gills of *M. galloprovincialis* exposed 4 weeks to Cd (0–20 µg/L), and

warming (25 °C), compared to control (20 °C, absence of Cd). Also no impacts on LPO levels were found in *Chamelea gallina* gills exposed for 7 days to increased temperatures (28 °C) while in the digestive gland of this clam LPO levels decreased compared to control temperature (22 °C) (Matozzo et al., 2013). Also, no cellular damages were observed in the scallop *Adamussium colbecki* after 2 weeks of exposure to Cd (0–40 μg/L) or warming conditions (Benedetti et al., 2016). Studies on the combined effects of metals and temperature also revealed different responses according to the species, exposure period, warming conditions and element tested. Attig et al. (2014) showed that LPO levels were higher when *M. galloprovincialis* was exposed for 4 days to Ni (13 μM) and augmented for increasing temperatures (18, 20, 22, 24, and 26 °C). Higher levels of LPO were also found when *A. colbecki* was exposed for 2 weeks to the combination of Cd and warming conditions (Benedetti et al., 2016). Recently Boukadida et al. (2017) showed no significant change in LPO in *M. galloprovincialis* larvae exposed for 48 h to Cu (6.67 μg/L) and different temperatures (18, 20, 22 °C).

When organisms are under adverse conditions, antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) activities may increase to eliminate the excess ROS produced and prevent cellular damage (Regoli and Giuliani, 2014). In the present study the activity of SOD was slightly enhanced when organisms were exposed to As and 21 °C apart after 28 days of exposure, but significantly higher activity was observed when both stressors were acting in combination (As 21 °C). A similar pattern was observed for CAT activity, with significantly higher values only observed in organisms exposed to the combination of As and temperature (As 21 °C), in this case both after 14 and 28 days of exposure. These results highlight the fact that the antioxidant capacity is time dependent and, although activated in the presence of As and warming (21 °C), this defense was only significantly increased, compared to control values, when As and warming were acting in combination, evidencing the impacts of the interaction between temperature and As on the antioxidant capacity of mussels. These findings may be related with higher As concentrations in organisms exposed to 21 °C in comparison to organisms under control temperature (17 °C), indicating that under warming conditions mussels were not able to prevent the entrance of As by closing their valves and therefore higher toxic impacts were generated by higher As bioconcentration due to warming conditions. Therefore, under As exposure and 21 °C the stress induced by As accumulated and temperature increase respectively, was not enough to significantly activate antioxidant enzymes. Our findings may further indicate that the increase of SOD and CAT activity in mussels exposed to the combination of As and temperature prevented greater cellular damage for this treatment, which was especially noticeable after 28 days of exposure. Assessing the impacts of metals in mussels, Duarte et al. (2011) observed the activation of SOD and CAT in *M. edulis* under an increasing gradient of Cd, Cu, and Zn. Benali et al. (2016) showed that CAT activity in *M. galloprovincialis* was negatively correlated with Cd and Cu concentrations. Higher CAT and SOD activity was also observed in clams contaminated with metals, including *R. decussatus* and *S. plana* under an environmental Hg contamination gradient (Ahmad et al., 2011; Velez et al., 2015b), and *R. decussatus* exposed for 96 h to Pb (0.1–1.8 mg/L; Freitas et al., 2014). Evaluating the effects of warming in mussels, Hu et al. (2015) showed that after 1, 3, 7 and 14 days of exposure *M. coruscus* antioxidant defenses at high temperature (30 °C) were significantly higher than those at the low temperature (25 °C). The activation of antioxidant enzymes was also observed in clams exposed to warming conditions. Velez et al. (2017) showed an induction of SOD in *R. decussatus* and *R. philippinarum* after 28 days of exposure to elevated temperature (25 °C) compared to control (17 °C); Matozzo et al. (2013) demonstrated that SOD activity was higher in the gills of *C. gallina* at 28 °C than at 22 °C; and Abele et al. (2002) identified an increase of SOD activity with a rise of temperature (0–22 °C, 4 weeks) in gill tissues of the mud clam *Mya arenaria*. When metals and warming conditions were tested in combination, studies conducted by Boukadida et al. (2017) showed that co-exposure to Cu and moderate elevated temperature (20 and 22 °C, 48 h) significantly increased the activity of catalase (CAT) in *M. galloprovincialis* larvae. However, Attig et al. (2014) demonstrated that, for the same mussel species, CAT activity was higher in organisms exposed to Ni at 18 and 20 °C in comparison to values recorded at 22, 24 and 26 °C. These authors also demonstrated that independently of the temperature tested, CAT activity values were maintained. Similarly, Nardi et al. (2017) showed that CAT activity was not changed in digestive glands and gills of *M. galloprovincialis* exposed to warming (25 °C), Cd and the combination of both stressors. Studying the impacts of temperature and Cd, Benedetti et al. (2016) showed that stressors acting alone had no effect on CAT activity while the combination of both stressors inhibited the activity of this enzyme in the digestive gland and gills of *A. colbecki*. Therefore, the mentioned studies and the present results may indicate that the activation of antioxidant enzymes depends on stress level, associated to metal concentration and temperature values, but may also depend on the period of exposure, tissue analyzed and the species used.

GSTs are a group of enzymes known for their ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification (Regoli and Giuliani, 2014). Previous studies conducted by Velez et al. (2016e) already demonstrated the role of these enzymes in As detoxification in bivalves. In the present study GSTs activity was significantly enhanced in mussels exposed to As, especially at higher temperatures (21 °C) where higher As concentrations were observed, showing the capacity of these enzymes to detoxify this metalloid. The increase of GSTs activity in mussels exposed to As may also contributed to prevent higher LPO in mussels exposed to As 17 °C and as well as in mussels exposed to the combined effect of As and temperature (As 21 °C). Furthermore, the significant increase observed in GSTs activity at As 21 °C after 28 days of exposure in comparison to values observed in mussels under the same condition but exposed for 14 days may be associated with the decrease of As concentrations at the end of the exposure period (28 days). Nevertheless, although higher GSTs activity was observed at As 21 °C than at As 17 °C, still higher As concentrations were observed in mussels exposed to the combination of both stressors. As mentioned previously, lower As concentrations in organisms exposed to 17 °C may result from the capacity of mussels to close their valves for longer periods when under control temperature (As 17 °C), corroborated by the fact that lower metabolic activity was observed at this condition, preventing As accumulation. Nevertheless, recent studies demonstrated that GSTs activity is metal dependent. Benali et al. (2016) demonstrated that Zn accumulation in *M. galloprovincialis* is positively correlated with GST activity, while Boukadida et al. (2017) showed that for the same species GSTs were not activated when mussels were exposed to Cu. The increase of GSTs activity when bivalves were exposed to metals was demonstrated in other bivalve species (Velez et al., 2015b, 2016e; Wang et al., 2012; Zhang et al., 2010). Concerning the effects of temperature on GSTs activity, Boukadida et al. (2017) showed that GSTs were higher at 20 and 22 °C compared to 18 °C in *M. galloprovincialis*. For the same mussel species Attig et al. (2014) showed that GSTs increased the activity with the increase of temperature up to 24 °C after which (26 °C) the activity decreased. Matozzo et al. (2013) showed higher GSTs activity in the gills of *C. gallina* at 28 °C compared to 22 °C. Assessing the combined effects of Cd and warming in GSTs activity, Benedetti et al. (2016) showed that Cd under control and warming conditions had no effect on GSTs activity but higher temperatures induced lower activity of this enzyme in the digestive gland of *A. colbecki*. On the other hand, these authors further observed that, for the same species but in the gills, Cd and warming acting alone had no effect on GSTs activity but increased activity of this enzyme was observed when both stressors were combined. Nardi et al. (2017) showed higher GSTs activity in digestive glands of *M. galloprovincialis* exposed to the combination of Cd and warming, followed by warming and Cd conditions. Recently Boukadida et al. (2017) demonstrated that the combination of Cu and temperature did not result in higher GSTs activity in *M. galloprovincialis*.

Regarding the impacts of As and warming on mussels metabolic performance, our study demonstrated that organisms exposed to As, 21 °C and As 21 °C decreased their metabolic capacity and increased their energy reserves in comparison to control organisms, which was time dependent. The metabolic capacity decrease may result from mussels valves closure and other behavioural adaptations to stress conditions (Anestis et al., 2007; Gosling, 2003). Among the biomarkers used to assess the impacts of pollutants and warming, the electron transport system (ETS) activity gives a proxy for the organisms' metabolic capacity (Bielen et al., 2016). It is important to note that although lower than at control conditions, the ETS activity was higher in mussels exposed to As 21 °C in comparison to mussels exposed to As and 21 °C separately, which may indicate that under the most stressful conditions mussels tried to adjust metabolic performance to fuel defence mechanisms, namely antioxidant enzymes that increased under this condition. This increased metabolism may be also responsible for higher As accumulation in mussels exposed to As 21 °C in comparison to As 17 °C. According to Anestis et al. (2007), *M. galloprovincialis* mussels exhibited a maximum period of valve opening when acclimated to temperatures between 10 °C and 17 °C and, in contrast, warming to 24 °C caused mussels to keep their valves closed for longer periods, which was accompanied by metabolic depression. Fanslow et al. (2001) further revealed that *Dreissena polymorpha* mussels showed increased ETS activity under environmental warming conditions.

Lower metabolism in mussels exposed to As and warming conditions resulted in the storage of energy reserves (GLY and PROT), especially at As 21 °C condition. Similar responses were observed in other bivalves exposed to metals, including the clam *Macoma balthica* that was also able to maintain GLY content under Cd contamination (10, 30, 100 µg/L) (Duquesne et al., 2004), while the clam *R. philippinarum* increased GLY content when exposed to As (2 mg/L) (Velez et al., 2016d). Studying the effects of Cd and warming in *M. galloprovincialis*, Nardi et al. (2017) further revealed that neutral lipids increase in mussels exposed to Cd, but also in response to warming and the combination of both stressors.

Regarding As accumulation the present study demonstrated that higher concentrations were observed at higher temperatures, both after 14 and 28 days of exposure. This situation is corroborated by lower metabolism observed at lower temperature (As 17 °C) in comparison to warming conditions (As 21 °C), which may indicate that mussels were able to close their valves for longer periods when under control temperature. Attig et al. (2014) also demonstrated that Ni concentrations increased in the digestive gland of *M. galloprovincialis* with the increasing temperatures (18, 20, 22, 24 °C) up to 26 °C where Ni concentration decreased. Nevertheless, opposite patterns were found in the same species exposed to other elements. Izagirre et al. (2014) demonstrated that in *M. galloprovincialis* Cd bioaccumulation was comparable at 18 and 26 °C after 24 h of exposure, and Nardi et al. (2017) observed for the same species no variation of Cd uptake for higher temperature (25 °C) after 4 weeks of exposure to Cd.

5. Conclusion

Overall our results demonstrated that both As and warming conditions induced oxidative stress and lowered the metabolism in *M. gallorprovincialis*, which was more pronounced when mussels were exposed to both stressors acting in combination. Thus, the present findings emphasize the importance of taking temperature into account when studying the effects of environmental toxicants such as As on bivalves, revealing that mussels subjected to one of the stressors in their natural environments (e.g. to elevated temperatures due to seasonal warming or extreme weather events) may become more susceptible to other stressors (such as trace element pollution) and vice versa, and emphasizes the importance of analyzing multiple stressors in estuaries.

Acknowledgments

Francesca Coppola and Ângela Almeida benefited from a Ph.D. Grants (SFRH/BD/118582/2016; SFRH/BD/86356/2012), Rosa Freitas and Bruno Henriques benefited from post-doc Grants (SFRH/BPD/92258/2013 and SFRH/BPD/112576/2015, respectively) given by the National Funds through the Portuguese Science Foundation (FCT), supported by FSE and Programa Operacional Capital Humano (POCH) e da União Europeia. Thanks are due, for the financial support to CESAM (UID/AMB/50017), to CIIMAR (UID/Multi/04423/2013), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. This work was supported by the Integrated Programme of SR & TD "Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate" (reference Centro-01-0145-FEDER-000018), co-funded by Centro 2020 program, Portugal 2020, European Union, through the European Regional Development Fund.

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